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**ARAŞTIRMA / RESEARCH**

# Role of nitric oxide in the regulation of pain after nociceptive stimuli in rat spinal cord

## Sıçan omuriliğinde nosiseptif uyaranlardan sonra ağrı düzenlemesinde nitrik oksidin rolü

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**Abstract**

**Purpose:** In this study, we examined the responses of the neurons in the lumbar segments of the spinal cord to nociceptive stimuli induced by formalin using c-fos immunohistochemistry and NADPH-d histochemistry

**Materials and Methods:** Thirteen male, Wistar albino rats were used. The rats were divided into 3 groups. Group 1: pain group (n=5). Group 2: sham group (n=5). Group 3: control group (n=3). In group 1, 50 µl of 30% formalin solution was injected subcutaneously unilaterally on the dorsal surface of the right foot. In group 2, phosphate buffered saline was injected subcutaneously unilaterally on the dorsal surface of the right foot. In group 3, no treatment was given. For each group lumbar spinal cord (SC) samples were taken. Samples were stained with c-fos and NADPH-d histochemistry and c-fos/NADPH-d double-labeling methods and evaluated under light microscope.

**Results:** We noted that cells on spinal cord sections stained with c-fos, NADPH-d and both became prominent with nociceptive stimuli. There were significant increases in the numbers of c-fos (+), NADPH-d (+) and double-labeled cells in pain group comparison to sham group. In group 1, NADPH was significantly increased in the Laminae I-IV of the lumbar segments of the SC on both sides and laminae VIII, IX and X on the same side.

**Conclusion:** The findings suggest that NO is associated with formalin-induced FOS-ir in the spinal cord, that it may play an important role in the regulation of pain and that the nociceptive function is related to increased nitric oxide synthase (+) neurons in the spinal cord.

**Key words:** Nitric oxide, pain, spinal cord

**Öz**

**Amaç:** C-fos indüksiyonunun, periferik inflamasyona karşı spinal kordda meydana gelen nöron yanıtının düzenlenmesinde önemli bir rol oynamaktadır. Sinir dokusunda NADPH-diaphorase (NADPH-d) reaksiyonu nitrik oksit sentaz (NOS) aktivitesinin iyi bir göstergesidir.

**Gereç ve Yöntem:** On üç erkek Wistar albino sıçan kullanıldı. Sıçanlar 3 gruba ayrıldı. Grup 1: ağrı grubu (n = 5). Grup 2: şam grup (n = 5). Grup 3: kontrol grubu (n = 3). Grup 1'de, 50 µl% 30 formalin solüsyonu, sıçan sağ ayağın dorsal yüzeyi üzerine tek taraflı subkutanöz enjekte edildi. Grup 2'de PBS, sıçan sağ ayağın dorsal yüzeyinde tek taraflı subkutanöz enjekte edildi. Grup 3'de ise tedavi verilmedi. Her grupta lumbar spinal cord (SC) örnekleri alındı. Numuneler c-fos immünohistokimyası, NADPH-d histokimyası ve c-fos / NADPH-d ikili etiketleme yöntemleriyle histokimyasal olarak boyandı ve ışık mikroskopu altında değerlendirildi.

**Bulgular:** Spinal kord kesitlerinde, hücrelerin c-fos, NADPH-d ile boyandığını ve her ikisinin de nosiseptif uyaranlarla belirginleştiğini saptadık. Ağrı grubunda, şam grubuna göre c-fos (+) (CF), NADPH-d (+) (NA) ve çift-etiketli (CN) hücre sayısında belirgin artış saptandı. Grup 1'de NADPH, spinal kord lomber segment kesitlerinin her iki yanındaki I-IV. laminalarında, aynı tarafta ise VIII, IX ve X laminalarında anlamlı olarak artmış halde bulundu.

**Sonuç:** Bulgular, NO'nun spinal kordda, formaline bağlı FOS-ir ile ilişkili olduğunu, ağrının düzenlenmesinde önemli bir rol oynayabileceğini ve nosiseptif fonksiyonun artmış nitrik oksit sentaz (+) nöronlarla ilişkili olduğunu göstermektedir.

**Anahtar kelimeler**: Nitrik oksit, ağrı, spinal kord.

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## INTRODUCTION

Transmission of sensorial information starts with the stimulation of the peripheral receptors of the first-order sensorial neurons whose bodies are located in the spinal ganglion. The central extensions of these neurons, which reside in the spinal ganglia, synapse with the second-order neurons in the dorsal horn of the spinal cord (SC). In the body, nitric oxide (NO) is an important physiological regulator of functions such as vasodilatation and neurotransmission[1]. Nitric oxide (NO) is a soluble gas continuously synthesized from the amino acid L-arginine in endothelial cells by the constitutive calcium calmodulin-dependent enzyme nitric oxide synthase (NOS)[2]. NO has a regulatory effect on the N-methyl-D-aspartate (NMDA) receptors of glutamate. Glutamate is the most predominant excitatory neurotransmitter involved in synaptic transmissionfrom primary sensory nerves to spinal dorsal horn neurons[3]. NOS immunocytochemistry of the tissues, in situ hybridization of NOS mRNA and reduced nicotinamide adenine dinucleotid phosphate diaphorase (NADPH-d) histochemistry have been used for defining of NO-producing neurons in the brain and spinal cord[4, 5]. Also, it has been shown that NO is released in different areas of the brain and spinal cord[2].

Fos is nuclear phosphoprotein product of c-fos proto-oncogene and is induced by peripheral stimuli. Fos is expressed rapidly and temporarily in the central nervous system as a response to injurious stimuli. Therefore, it is used as a neuronal marker[6]. Together with NADPH-d reactive cells, Fos reactive neurons are distributed in the zona marginalis and the neck of the dorsal horn of the SC[7]. It has been shown that early-response genes such as c-fos is expressed in the SC by peripheral inflammation[6]. It has been argued that induction of c-fos plays a significant role in regulation of neuronal response to peripheral inflammation[6].

In this study, we examined the responses of the neurons in the lumbar segments of the SC to nociceptive stimuli induced by formalin using c-fos immunohistochemistry and NADPH-d histochemistry.

## MATERIALS AND METHODS

In the present study, 13 adult, male, Wistar albino rats (300±-50 g) were used. Rats were purchased from the Experimental Animal Research Laboratory of Ege University. The rats were kept under a temperature of 22°C, humidity of 65%, and light–dark cycles of 12:12 h, and they had ad libitum access to laboratory standard food and water. This study was approved by The Animal Experiments Local Ethics Committee of Manisa Celal Bayar University, Turkey.

The rats were grouped as follows:

- Group 1: pain group (n=5), 50 µl of 30% formalin solution was injected subcutaneously unilaterally on the dorsal surface of the right foot.
- Group 2: sham group (n=5), phosphate buffered saline (PBS) was injected subcutaneously unilaterally on the dorsal surface of the right foot.
- Group 3: control group (n=3) no treatment was given.

An hour after unilateral injection of formalin, rats were sacrificed under anesthesia and lumbar SC samples were taken. Samples were stained with histochemical and immunohistochemical methods and evaluated under light microscope.

### Histochemical analysis

The study included formalin-fixed, paraffin-embedded samples from control and experimental groups. Routine paraffin embedding procedures were used. In brief, tissue samples were fixed in 10% formalin solution, dehydrated in graded ethanol series, cleared in xylene, embedded in paraffin and 5 µm thick sections were cut. The tissue blocks were chosen carefully after histological assessment of sections stained with hematoxylin and eosin (H & E). Selected sections were stained immunohistochemically. For immunohistochemical staining, sections were incubated at 60°C overnight and then cleared in xylene for 30 min. After washing with a decreasing series of ethanol, sections were washed with distilled water and phosphate-buffered saline (PBS) for 10 min. Sections were then treated with 2% trypsin in Tris buffer (50 mM Tris base and 150 mM NaCl dissolved in deionized $H_2O$) at $37^0$C for 15 min and washed with PBS. Sections were delineated with a Dako pen (Dako, Glostrup, Denmark) and incubated in a solution of 3% $H_2O_2$ for 15 min to inhibit endogenous peroxidase

activity. Then sections were washed with PBS and incubated for 18 h at 4°C with primary antibody: anti-collagen type-I (mouse monoclonal type IC, 2456-Sigma,USA) in a 1:100 dilution. Afterwards, sections were washed 3 times for 5 min each with PBS, followed by incubation with biotinylated secondary antibody and then with streptavidin conjugated to horseradish peroxidase in PBS for 30 min each (Zymed Histostain-plus-Peroxidase- kit, 85-9043, San Francisco, CA). After washing 3 times with PBS, sections were incubated with DAB substrate containing diaminobenzidine (5 min) to stain immunolabelling and then with Mayer's hematoxylin. Sections were covered with mounting medium and were analyzed with an Olympus BX 40 brightfield microscope (Olympus, Tokyo, Japan).

### Immunohistochemical analysis

An hour after unilateral injection of formalin, rats were sacrificed under anesthesia and lumbar SC samples were fixed in 4% paraformaldehyde for 4 hours. Samples were left in 30% sucrose solution to precipitate and stored after freezing. Free-floating sections of 25 μm thickness were taken from the samples using cryostat. Sections were stained for fos immunohistochemistry (primary antibody rabbit c-fos 1:2000, secondary antibody biotinylated anti-rabbit IgG 1:200, Oncogene Research Products, Cambridge, MA, USA). Sections were then treated with hydrogen peroxide to make the reaction visible and DAB was used as chromogene for 20 minutes. Sections were treated for NADPH-d histochemical reaction (0.1 mg/ml nitroblue tetrazolium and 0.5 mg/ml NADPH tetrasodium salt) for 30 minutes, washed with phosphate buffer and incubated at $37^0$C for 30 minutes using 0.3% Triton X-100, 0.5 mg/ml nitroblue tetrazolium and 0.1 M phosphate buffer (pH 7.3) containing 1 mg/ml NADPH tetrasodium salt. Sections were stained, placed on glass, viewed under light microscope (Olympus BX40, Tokyo, Japan) and images were obtained by a digital camera. Using the digital images, the numbers of c-fos (+), NADPH-d (+) and double-labeled cells in the Rexed laminae of the SC were counted and their morphologies were evaluated[8].

### Statistical analysis

Immunohistochemical intensity was scored by two blinded observers as follows: 0, no staining; 1, weak staining; 2, moderate staining; 3, moderate–strong staining, 4, strong staining, and 5, very strong staining. Histological results were calculated on GraphPad (GraphPad Software, San Diego, CA, USA) using one-way analysis of variance and are presented as mean ± standard deviation. Statistical significance was defined as $P \leq 0.05$.

## RESULTS

### Light microscopy

Formalin was injected subcutaneously on the dorsal surface of the right feet of rats and c-fos and NOS were rendered visible in the neurons of the lumbar segments of the spinal cord by using specific antibodies and NADPH-d reaction, respectively (Fig. 1). It was noted that cells on SC sections stained with c-fos, NADPH-d and both became prominent with nociceptive stimuli (Fig. 2, 3). There were significant increases ($p<0.001$) in the numbers of c-fos (+) (CF), NADPH-d (+) (NA) and double-labeled (CN) cells in comparison to the basal levels observed in the sham group (Fig. 2). The number of stained cells was significantly greater in the lower lumbar segments ($p<0.05$). Significant laterality difference existed for c-fos ($p<0,05$) but not NADPH-d. The number of stained cells was higher in Laminae I, II, III and IV (Fig 4 and Fig 5).

Nociceptive stimuli induce increases in the expressions of c-fos and NADPH, especially by neurons of the superficial laminae of the dorsal horn. In pain group, NADPH was significantly increased in the Laminae I-IV of the lumbar segments of the SC on both sides and laminae VIII, IX and X on the same side ($p<0.001$). There were no significant differences in the other laminae ($p>0.05$). In pain group, NADPH-d reaction was bilateral in the dorsal horn while c-fos induction was localized to laminae I-IV, VIII and IX of the same side ($p<0.05$) only (Fig 4. and Fig 5.).

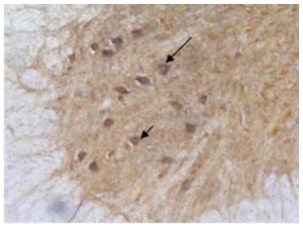

**Figure 1. Double-labeled (long arrow) and c-fos-ir cell (short arrow) (X20).**

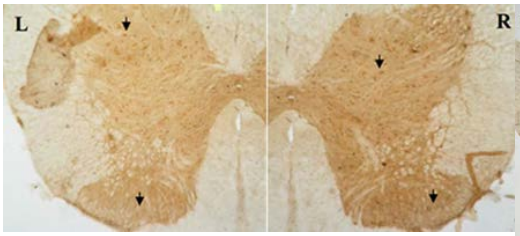


**Figure 2. Cross-section of the SC of a rat without formalin-induced pain; basal levels of NADPH-d reaction and c-fos (+) cells can be seen (X20).**

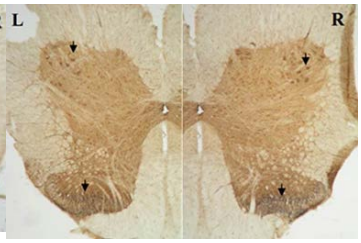


**Figure 3. Cross-section of the SC of a rat with formalin-induced pain; bilaterally increased NADPH-d reaction and increased c-fos (+) cells can be observed, more prominently on the right side (R) (X20)**

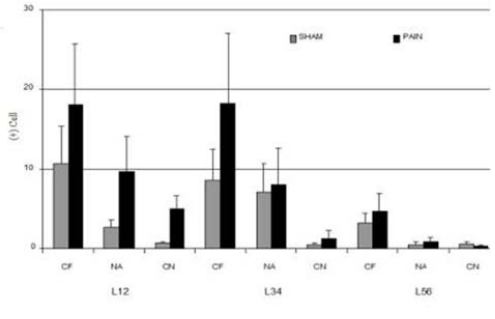


**Figure 4. Distribution of c-fos (+) (CF), NADPH-d (+) (NA) and double-labeled (CN) cells with respect to the SC laminae in the sham and formalin-induced pain groups.**

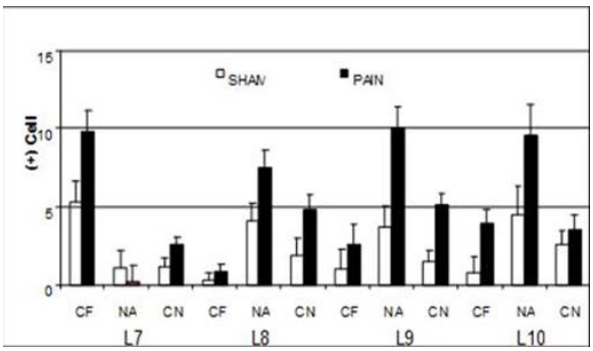


**Figure 5. Distribution of c-fos (+) (CF), NADPH-d (+) (NA) and double-labeled (CN) cells with respect to the SC laminae in the sham and formalin-induced pain groups.**

## DISCUSSION

Pain receptors/nociceptors relay stimuli in the form of electrical impulse to the SC via nerves. C-fos, the protein product of the immediate early gene c-fos, has long been known as a marker for the activation of nociceptive neurons in the spinal and medullary dorsal horns. It has been shown that induction of c-Fos in the spinal and medullary dorsal horns is enhanced in response to stimulation of the surrounding areas innervated by the injured nerve[9]. Therefore, demonstration of fos expression is regarded as a reliable marker in defining the neurons after nociceptive stimuli.

Motojima Y et al. injected formalin subcutaneously bilaterally into the hind paws of adult male c-fos-eGFP transgenic or wild type rats and authors demonstrated Fos-like immunoreactivity neurons[10]. Fos expression can also be used to demonstrate the neurons in vascular occlusion or withdrawal abstinence pain[11]. Blomqvist and Craig reported that, fos expression clearly depicts activation of the neurons in the superficial "pain-processing" laminae by innocuous stimuli after nerve lesions which is called as "allodynia"[12]. Nociceptive stimuli and acute inflammation have been shown to induce fos expression and increase NADPH in the SC [13,14].

Barr showed that NO induced fos expression in neighboring neurons[15]. NO, by activating guanyl cyclase in both intracellular and extracellular pathways can act on fos neurons [16]. NO does not induce fos expression in all NADPH-d (+) neurons of the SC. Fos expression shows neuronal activation in response to nociceptive stimulus. Absence of NOS (+) neurons does not mean that neurons are

not activated and it is also true that they may not express a gene even if all neurons are activated[12] .NOS immunoreaction and NADPH-d reaction are consistent with the distribution of labeled neurons in the dorsal horn. Large motor neurons and lateral spinal nuclei that show marked NOS immunoreaction but not NADPH-d reaction may be observed. It has been shown, using double-labeling, that NADPH-d (+) neurons in the dorsal horn were closely related to nerve fibers that show substance P and CGRP immunoreactivity[17]. Double-labeling not only shows the close relation between numerous fos-labeled neurons and NADPH-d labeled nerve fibers but also suggests that a functional relationship exists between the expression of transcription factors that encode early-response gene in rat SC and the presence of NO[18]. Neurons of adult rats that are NOS (+) and/or show mild NADPH-d activity are located in the laminae I and II (substantia gelatinosa) of the dorsal horn of the SC. These laminae of the SC are known to modulate the nociceptive stimuli arising from the periphery[19].

In the light of this information, mapping of fos expression in the dorsal horn would be helpful for defining the anatomy of the nociceptive system and also to asses the efficacies of analgesic treatments. In present study, c-fos immunoreactivity and NADPH-d reaction were minimal at laminae I, II, III and X in sham group. However, c-fos and NADPH-d positive neurons in the superficial laminae of the dorsal horn (especially L3-6 segments of the spinal cord on the same side) were significantly increased in the pain group ($p<0.05$). C-fos (+) neurons were found in laminae I-IV and X on the same side while NADPH-d reaction was induced bilaterally. There was a few c-fos (+) neurons on contralateral SC laminae. Moreover, number of NADPH-d (+) neurons were higher than of c-fos (+) neurons. Also numerous fos/NADPH-d double-labeled neurons (densely stained NADPH-d (+) perikarya and cytoplasmic elongations adjacent to or in close proximity to fos (+) nuclei) were demonstrated.

C-fos expression can be detected in the ipsilateral SC 30 minutes after formalin injection, reaches its peak at 2 hours and is predominantly localized to laminae I-II, III-IV and X. Only a small number of labeled neurons can be observed in the contralateral SC. Increased intracellular $Ca^{+2}$ induce c-fos expression[20]. In present study, bilateral increase in NADPH especially in laminae I-IV of the lumbar segments of the SC and ipsilateral increase in NADPH-d in laminae VIII, IX and X were observed. This response is in contrast with ipsilateral c-fos induction. This led us to believe that formalin-induced inflammation results in NADPH signal activation in nociceptive neurons of the SC through a mechanism mediated by NMDA receptors and that this signal can trigger hyperalgesia. It is known that c-fos induction by NADPH and possibly some late-response genes play important roles in response to peripheral hyperalgesia. Since NADPH induction is more widespread and sensitive, it can be a better indicator of neuronal activity following nociceptive stimuli than c-fos expression. The limitation of the studyis that we did not use immunohistochemistry and neuronal nos staining.

The results of the present study showed that dorsal horn neurons of the lumbar segments of the SC contained fos and/or NADPH-d and NADPH-d (+) perikarya and cytoplasmic extensions are closely related to fos immunoreactive nuclei in many neurons. This relation lend support to the hypothesis that NO plays a role in modulation pain. However, further clinical and experimental studies are needed to identify NO's role in pain regulation.

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